



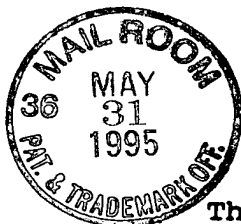
# APPLICATION FOR UNITED STATES PATENT

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Invention: DNA ENCODING A GROWTH FACTOR SPECIFIC FOR  
EPITHELIAL CELLS

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## SPECIFICATION

FIELD OF THE INVENTION

5 The present invention relates to growth factors, particularly to isolation of a polypeptide growth factor similar to a family of factors including known fibroblast growth factors (FGFs). This invention also relates to construction of complementary DNA (cDNA) segments from messenger RNA (mRNA) encoding the novel growth factor. Further, this invention pertains to synthesis of products of such DNA segments by recombina-  
10 tant cells, and to the manufacture and use of certain other novel products enabled by the identification and cloning of DNAs encoding this growth factor.

ABBREVIATIONS USED IN THIS APPLICATION

	aFGF	acidic fibroblast growth factor
	bFGF	basic fibroblast growth factor
	EGF	epidermal growth factor
5	HSAC	heparin-Sepharose affinity chromatography
	kb	kilobases
	kDa	kilodaltons
	KGF	keratinocyte growth factor
10	NaDodSO <sub>4</sub> /PAGE	Sodium dodecylsulphate (SDS)/ polyacrylamide gel electrophoresis
	RP-HPLC	reversed-phase high performance liquid chromatography
	TGF $\alpha$	transforming growth factor $\alpha$

## BACKGROUND OF THE INVENTION

Growth factors are important mediators of intercellular communication. These potent molecules are generally released by one cell type

5 and act to influence proliferation of other cell

types <sup>James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem.</sup> ~~(see reference I-1 in Experimental Section~~

~~53, 259-292~~

~~I, below)~~. Interest in growth factors has been

heightened by evidence of their potential

involvement in neoplasia <sup>Sporn, A.B. and Todaro,</sup> ~~(reference II-2 in~~

~~M.J., 1980, N. Eng. J. Med. 303, 878-880~~

~~Experimental Section II, below)~~. The v-sis

transforming gene of simian sarcoma virus encodes

a protein that is homologous to the B chain of

<sup>James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292</sup> platelet-derived growth factor ~~(I-1, I-2)~~

Moreover, a number of oncogenes are homologues of

<sup>James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292</sup> genes encoding growth factor receptors ~~(I-1)~~.

Thus, increased understanding of growth factors

and their receptor-mediated signal transduction

pathways is likely to provide insights into

mechanisms of both normal and malignant cell

20 growth.

One known family of growth factors

affecting connective tissue cells includes acidic

fibroblast growth factor (aFGF), basic fibroblast

growth factor (bFGF), and the related products of

<sup>C</sup> ~~the hst and int-2 oncogenes.~~ <sup>FGF-5</sup>

Further, it is known that some growth factors, including the following, have heparin-

sub  
35      sub  
36

binding properties: aFGF (~~I-20~~, ~~I-21~~); bFGF (~~I-~~ <sup>sub 34</sup>

<sup>sub 33</sup>  
~~19~~, ~~I-20~~); granulocyte/macrophage colony

B James R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292  
stimulating factor (~~I-1~~); and interleukin 3 (~~I-~~

B James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292  
~~1~~). Each of these polypeptide factors is

B 5 James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292 <sup>sub 31</sup> <sup>sub 32</sup>  
produced by stromal cells (~~I-1~~, ~~I-2~~, ~~I-25~~). Such

factors appear to be deposited in the

James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292 <sup>sub 33</sup>  
B extracellular matrix, or on proteoglycans coating  
the stromal cell surface (~~I-1~~, ~~I-25~~). It has

been postulated that their storage, release and  
10 contact with specific target cells are regulated  
by this interaction (~~I-25~~, ~~I-28~~). <sup>sub 35</sup> <sup>sub 36</sup>

It is widely recognized, however, that

the vast majority of human malignancies are  
B Wright, N. and Allison, M., 1984, The Biology of Epithelial Cell Populations [Oxford University Press, New York] Vol.  
derived from epithelial tissues (~~I-5~~). Effectors 1, pp. 3-5

15 of epithelial cell proliferation derived from  
B <sup>mesenchymal cells</sup> James, R. and Bradshaw, R.A., 1984, Ann. Rev. Biochem. 53, 259-292  
mesenchymal tissues have been described (~~I-1~~, ~~I-~~  
Waterfield, M.D., et al., 1983, Nature 304, 35-39  
B <sup>sub 31</sup> ~~2~~, ~~I-3~~), however, their molecular identities and  
structures have not been elucidated.

In light of this dearth of knowledge  
20 about such mesenchymal growth factors affecting  
epithelial cells, it is apparent that there has  
been a need for methods and compositions and  
bioassays which would provide an improved  
knowledge and analysis of mechanisms of  
25 regulation of epithelial cell proliferation, and,  
ultimately, a need for novel diagnostics and  
therapies based on the factors involved therein.

This invention contemplates the application of methods of protein isolation and recombinant DNA technologies to fulfill such needs and to develop means for producing protein factors of mesenchymal origin, which appear to be related to epithelial cell proliferation processes and which could not be produced otherwise. This invention also contemplates the application of the molecular mechanisms of these factors related to epithelial cell growth processes.

## SUMMARY OF THE INVENTION

The present invention relates to developments of protein isolation and recombinant DNA technologies, which include production of novel growth factor proteins affecting epithelial cells, free of other peptide factors. Novel DNA segments and bioassay methods are also included.

The present invention in particular relates to a novel protein having structural and/or functional characteristics of a known family of growth factors which includes acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF) and the related products of the <sup>FGFs</sup> *hst*<sub>1</sub> and *int-2* oncogenes. This new member of the FGF polypeptide family retains the heparin-binding properties of the FGFs but has evolved a unique target cell specificity. This growth factor appears to be specific for epithelial cells and is particularly active on keratinocytes. Therefore, this novel factor has been designated "keratinocyte growth factor" (KGF). Notwithstanding its lack of activity on fibroblasts, since it is the sixth known member of the FGF polypeptide family, KGF may also be referred to as FGF-6.

Accordingly, this invention relates, in part, to purified KGF or KGF-like proteins and

methods for preparing these proteins. Such purified factors may be made by cultivation of human cells which naturally secrete these proteins and application of isolation methods according to the practice of this invention. These proteins can be used for biochemical and biological studies leading, for example, to isolation of DNA segments encoding KGF or KGF-like polypeptides.

10                   The present invention also relates to such DNA segments which encode KGF or KGF-like proteins. In a principal embodiment, the present invention relates to DNA segments, which encode KGF-related products, consisting of: human cDNA clones 32 or 49, derived from polyadenylated RNA extracted from the human embryonic lung fibroblast cell line M426; recombinants and mutants of these clones; and related DNA segments which can be detected by hybridization to any of the above human DNA segments, which related segments encode KGF-like proteins or portions thereof.

20                   In the practice of one embodiment of this invention, the DNA segments of the invention are capable of being expressed in suitable host cells, thereby producing KGF or KGF-like proteins. The invention also relates to mRNAs produced as the result of transcription of the



sense strands of the DNA segments of this invention.

5 In another embodiment, the invention relates to a recombinant DNA molecule comprising a vector and a DNA of the present invention. These recombinant molecules are exemplified by molecules comprising a KGF cDNA and any of the following vector DNAs: a bacteriophage  $\lambda$  cloning vector (exemplified by  $\lambda$ pCEV9); a DNA sequencing  
10 plasmid vector (e.g., a pUC variant); a bacterial gene expression vector (e.g., pKK233-2); or a mammalian gene expression vector (such as pMMT).

In still another embodiment, the invention comprises a cell, preferably a  
15 mammalian cell, transformed with a DNA of the invention. Further, the invention comprises cells, including insect cells, yeast cells and bacterial cells such as those of *Escherichia coli* and *B. subtilis*, transformed with DNAs of the invention. According to another embodiment of this aspect of  
20 the invention, the transforming DNA is capable of being expressed in the cell, thereby increasing in the cell the amount of KGF or KGF-like protein encoded by this DNA.

25 The primary KGF translation product predicted from its cDNA sequence contains an N-terminal hydrophobic region which likely serves

as a signal sequence for secretion and which is not present in the mature KGF molecule. In a most preferred embodiment of the gene expression aspect of the invention, the cell transformed by the DNA of the invention secretes the protein encoded by that DNA in the (truncated) form that is secreted by human embryonic lung fibroblast cells.

Still further, this invention contemplates KGF or KGF-like proteins produced by expression of a DNA of the invention, or by translation of an RNA of the invention. Preferably, these proteins will be of the secreted form (i.e., lacking an apparent signal sequence). These protein factors can be used for functional studies, and can be purified for additional structural and functional analyses, such as qualitative and quantitative receptor binding assays.

Moreover, the ability to produce large quantities of this novel growth factor by recombinant techniques will allow testing of its clinical applicability in situations where specific stimulation of growth of epithelial cells is of particular importance. Accordingly, this invention includes pharmaceutical compositions comprising KGF or KGF-like polypeptides for use in the treatment of such

conditions, including, for example, healing of wounds due to burns or stimulation of transplanted corneal tissue.

5           According to this embodiment of the invention, the novel KGF-like proteins will be protein products of "unmodified" DNAs and mRNAs of the invention, or will be modified or genetically engineered protein products. As a result of engineered mutations in the DNA  
10 sequences, modified KGF-like proteins will have one or more differences in amino acid sequence from the corresponding naturally occurring "wild-type" proteins. According to one embodiment of this aspect of this invention, the modified KGF-  
15 like proteins will include "chimeric" molecules comprising segments of amino acid sequences of KGF and at least one other member of the FGF peptide family.

          Ultimately, given results of analogous  
20 successful approaches with other peptide factors having similar properties, development of such chimeric KGF-like polypeptides should lead to superior, "second generation" forms of KGF-like peptides for clinical purposes. These modified  
25 KGF-like products might be smaller, more stable, more potent, and/or easier or less expensive to produce, for example.

This invention further comprises novel bioassay methods for determining expression in human cells of the mRNAs and proteins produced from the genes related to DNA segments of the invention. According to one such embodiment, DNAs of this invention may be used as probes to determine steady state levels or kinetics of induction of related mRNAs. The availability of the KGF-related cDNA clones makes it possible to determine whether abnormal expression of this growth factor is involved in clinical conditions characterized by excessive epithelial cell growth, including dysplasia and neoplasia (e.g., psoriasis, or malignant or benign epithelial tumors).

This invention also contemplates novel antibodies made against a peptide encoded by a DNA segment of the invention. In this embodiment of the invention, the antibodies are monoclonal or polyclonal in origin, and are generated using KGF-related polypeptides from natural, recombinant or synthetic chemistry sources.

The antibodies of this invention bind specifically to KGF or a KGF-like protein which includes the sequence of such peptide, preferably when that protein is in its native (biologically active) conformation. These antibodies can be used for detection or purification of the KGF or

~~antibodies can be used for detection or~~  
~~purification of the KGF or KGF-like protein~~  
factors. In a most preferred embodiment of this  
aspect of the invention, the antibodies will  
5 neutralize the growth promoting activity of KGF,  
thereby enabling mechanistic studies and,  
ultimately, therapy for clinical conditions  
involving excessive levels of KGF.

BRIEF DESCRIPTION OF THE DRAWINGS

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SUB  
FO  
Fig. 1-1 depicts results of heparin-  
Sephadex affinity chromatography of conditioned  
medium from M426 human embryonic fibroblasts  
5 showing that greater than 90% of the mitogenic  
activity for mouse keratinocytes (BALB/MK) eluted  
with 0.6 M NaCl.

Fig. 1-2<sup>A, 2B and 2C</sup> illustrates results of further  
purification of the mitogen from human  
fibroblasts using HPLC with an adsorptive  
matrix. Panel (A) shows the profile on reversed-  
phase (C<sub>4</sub>) HPLC of BALB/MK mitogenic activity.  
Panel (B) presents electrophoretic (NaDodSO<sub>4</sub>/PAGE)  
analysis of selected fractions from the C<sub>4</sub>  
15 chromatography shown in panel A, demonstrating  
that the peak HPLC fractions contained a single  
band on the silver stained gel. Panel (C) is a  
bar graph of DNA synthesis in BALB/MK cells  
triggered by the fractions analyzed in Panel B,  
20 showing that the relative mitogenic activity  
correlated well with the intensity of the protein  
band across the activity profile.

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Fig. 1-3 presents an alternative  
purification step to RP-HPLC, using sieving  
chromatography with a (TSK G3000SW GlasPac)  
25 column run in aqueous solution near physiologic

pH, which resulted in a major peak of mitogenic activity in the BALB/MK bioassay.

Fig. I-4 illustrates a comparison of BALB/MK DNA synthesis in response to TSK-purified mitogen and other growth factors.

Fig. I-5 shows comparisons of growth of BALB/MK cells in a chemically defined medium in response to different combinations of growth factors.

Table I-1 summarizes the results from various purification steps, documenting that sieving chromatography provided a far better recovery of activity than the adsorptive RP-HPLC approach.

Table I-2 recapitulates data on the target cell specificities of various growth factors, demonstrating that the newly isolated factor exhibited a strong mitogenic effect on keratinocytes (BALB/MK) and, in striking contrast, had no detectable effects on fibroblasts or human saphenous vein endothelial cells.

Fig. II-1 presents the nucleotide sequence and deduced amino acid sequence of KGF cDNA, as well as identification of RNAs transcribed from the KGF gene. Panel (A) outlines a schematic representation of human KGF cDNA clones. Panel (B) documents the KGF cDNA

nucleotide and predicted amino acid sequences.

(C) Identification of RNA transcripts of KGF genes by Northern blot analysis.

Fig. II-2 illustrates the topological comparison of the FGF family of related molecules, including KGF, with emphasis on the two protein domains that share high homology, the putative signal peptide sequences, and the two conserved cysteine residues.

Fig. II-3 shows (Northern blot) analyses

of expression of KGF-related mRNA in selected normal human cell lines and tissues, revealing

that a single 2.4 kb transcript was present in

RNA from human embryonic lung fibroblasts and

from adult skin fibroblasts, while no transcript

was detected in the (B5/589) epithelial or (HA83)

glial cell lines, or in primary cultures of human

saphenous vein endothelial cells.

Table II-1 summarizes a comparison of the effect of heparin on KGF mitogenic activity with effects on other growth factors, showing that thymidine incorporation into DNA by BALB/MK cells in response to KGF was inhibited by heparin, in contrast, to the activities of both aFGF and bFGF which were increased by the same treatment.



## DESCRIPTION OF SPECIFIC EMBODIMENTS

This invention relates, in part, to purified KGF or KGF-like proteins and methods for preparing these proteins. A principal embodiment of this aspect of this invention relates to homogeneous KGF characterized by an apparent molecular weight of about 28 kDa based on migration in NaDodSO<sub>4</sub>/PAGE, movement as a single peak on reversed-phase high performance liquid chromatography, and a specific activity of at least about  $3.4 \times 10^4$  units per milligram, and preferably at least about  $3.2 \times 10^5$  units per milligram, where one unit of activity is defined as that amount which causes half of the maximal possible stimulation of DNA synthesis in certain epithelial (keratinocyte) cells under standard assay conditions outlined below.

To identify novel growth factors specific for epithelial cell types, a clonal BALB/c mouse keratinocyte cell line, designated

B *Weissman, B.E. and Aaronson, S.A., 1983, Cell 32, 599-606*  
BALB/MK (~~1-6~~) was employed as an indicator cell to detect such factors. These cells are dependent for their growth upon an exogenous

source of an epithelial cell mitogen even in

B 25 *Weissman, B.E. and Aaronson, S.A., 1983, Cell 32, 599-606*  
medium containing serum (~~1-6~~). The development of chemically defined medium for these cells has made it possible to demonstrate that two major

mitogenic pathways are required for BALB/MK proliferation. One involves insulin-like growth factor I (or insulin at high concentration) and the other is satisfied by epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF $\alpha$ ), acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF) (1-7).  
*Falco, J.P., et al., 1988, Embryone 2, 573-578*

By using BALB/MK as the prototypical epithelial cell line and NIH/3T3 as its fibroblast counterpart, conditioned media from various human cell lines were assayed for new epithelial cell-specific mitogens. These bioassays of this invention enabled the purification to homogeneity of one such novel growth factor, released by a human embryonic lung fibroblast line, and designated herein as keratinocyte growth factor (KGF).

In brief, the bioassay for KGF-like activity under standard conditions comprises the following steps:

- (i) Mouse keratinocytes (BALB/MK cells) are grown in culture to confluency and then maintained for 24-72 hr in serum-free medium;
- (ii) Following addition of test samples, stimulation of DNA synthesis is determined by incorporation of  $^3\text{H}$ -thymidine into acid-precipitable DNA.

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To determine the <sup>target</sup> cell target specificity of a mitogenic growth factor, the DNA synthesis stimulation, expressed as ratio of stimulated synthesis over background incorporation of thymidine in the absence of added test sample, can be compared to analogous stimulation observed in cells other than keratinocytes under the same assay conditions. In such comparisons, KGF mitogenic activity will exhibit marked specificity for the keratinocytes as opposed to fibroblasts (at least about 500-fold greater stimulation) and lesser but significant (at least about 50-fold) greater activity on keratinocytes than on other exemplary epithelial cell types (see Table T-2 for further data, and Materials and Methods in Experimental Section I for details of the standard conditions of the bioassay).

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By employing a method of KGF production involving culturing cells and isolating mitogenic activity, which method comprises ultrafiltration, heparin-Sepharose affinity chromatography (HSAC) and adsorptive reversed-phase high performance liquid chromatography (RP-HPLC) or, alternatively, molecular sieving HPLC (TSK-HPLC), according to the present invention, a quantity was isolated sufficient to permit detailed characterization of the physical and biological properties of this molecule.

To summarize, the method for production of KGF from producing cells such as M426 human embryonic fibroblasts (<sup>sub</sup>~~I-8~~), for example, comprises the following steps:

- 5           (i) Preparation of conditioned media (e.g., 10 liters) using monolayer cultures cycled from serum-containing to serum-free medium and storing the serum-free harvest at -70°C until further use;
- 10          (ii) Concentration by ultrafiltration using membranes having a 10 kDa molecular weight cutoff in several successive steps with intervening dilution in buffer (to facilitate removal of low molecular weight materials), followed by optional  
15 storage at -70°C;
- (iii) Affinity chromatography on heparin attached to a polymeric support (e.g., Sepharose) with elution by a gradient of increasing NaCl concentration;
- 20          (iv) Concentration by a factor of at least ten- to twenty-fold with small scale ultrafiltration devices with a 10 kDa molecular weight cutoff (e.g., a Centricon-10 microconcentrator from Amicon) and storage at  
25 -70°C.

The next step of the purification process comprises either step (v) or, alternatively, step (vi), as follows:

(v) Reversed-phase HPLC of active fractions (0.6 M NaCl pool) from the previous HSAC step in organic solvent systems;

or,

5        (vi) Molecular sieve HPLC (e.g, on a TSK-G3000SW Glas-Pac Column from LKB) in aqueous buffer at near physiological pH (e.g., Tris-HCl, pH 6.8/0.5M NaCl) followed by storage at -70°C.

F  
10        A preparation made by the TSK step (vi) was almost as pure as one obtained from RP-HPLC, as judged by silver-stained NaDodSO<sub>4</sub>/PAGE (data not shown); but the TSK approach provided a far better recovery of activity (Table 1).  
15        Further, the TSK-purified material had a higher specific activity than the RP-HPLC material. KGF prepared by the TSK procedure above stimulated DNA synthesis in epithelial cells at sub-nanomolar concentrations, but failed to induce any thymidine incorporation into DNA of  
20        fibroblasts or endothelial cells at comparable or higher concentrations (up to 5 nM). The activity was sensitive to acid, heat and solvents used in the RP-HPLC step. (See Experimental Section I for data on sensitivities and further details of the  
25        production method.)

Using standard methodology well known in the art, an unambiguous amino acid sequence was determined for positions 2-13 from the amino

terminus of the purified KGF, as follows: Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala-Thr-Asn-Val (see Experimental Section I).

5 The present invention also includes DNA segments encoding KGF and KGF-like polypeptides. The DNAs of this invention are exemplified by . DNAs referred to herein as: human cDNA clones 32 and 49 derived from polyadenylated RNA extracted from the human embryonic lung fibroblast cell  
10 line M426; recombinants and mutants of these clones; and related DNA segments which can be detected by hybridization to these DNA segments.

As described in Experimental Section II, to search for cDNA clones corresponding to the  
15 known portion of the KGF amino acid sequence, two pools of oligonucleotide probes were generated based upon all possible nucleotide sequences encoding the nine-amino acid sequence, Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala. A cDNA  
20 library was constructed in a cDNA cloning vector,  $\lambda$ pCEV9, using polyadenylated RNA extracted from the human embryonic lung fibroblast cell line M426 which was the initial source of the growth factor. Screening of the library ( $9 \times 10^5$   
25 plaques) with the  $^{32}\text{P}$ -labelled oligonucleotides identified 88 plaques which hybridized to both probes.

Of 10 plaque-purified clones that were analyzed, one, designated clone 49, had a cDNA insert of 3.5 kb, while the rest had inserts ranging from 1.8 kb to 2.1 kb. Analysis of the smaller clones revealed several common restriction sites, and sequencing of a representative smaller clone, designated clone 32, along with clone 49, demonstrated that they were overlapping cDNAs (Fig ~~II-1A~~<sup>b</sup><sub>a</sub>). Alignment of the two cDNAs established a continuous sequence of 3.85 kb containing the complete KGF coding sequence. The sense strand DNA nucleotide sequence, and the predicted primary protein sequence encoded, are shown for the full-length composite KGF cDNA sequence in Fig. ~~II-1B~~<sup>7</sup><sub>a</sub>.

These DNAs, cDNA clones 32 and 49, as well as recombinant forms of these segments comprising the complete KGF coding sequence, are most preferred DNAs of this invention.

From the cDNA sequence, it is apparent that the primary KGF<sub>a</sub><sup>FGF-5</sup> and hsf translation products contain hydrophobic N-terminal regions which likely serve as signal sequences, based on similarity to such sequences in a variety of other proteins. Accordingly, this N-terminal domain is not present in the purified mature KGF molecule which is secreted by human embryonic fibroblasts.

Furthermore, KGF shares with all other members of the FGF family two major regions of homology, spanning amino acids 65-156 and 162-189 in the predicted KGF sequence, which are  
5 separated by short, nonhomologous series of amino acids of various lengths in the different family members. The sequence of the purified form of KGF contains five cysteine residues, two of which are conserved throughout the family of FGF  
10 related proteins. Five pairs of basic residues occur throughout the KGF sequence. This same pattern has been observed in other FGF family members.

It should be obvious to one skilled in  
15 the art that, by using the DNAs and RNAs of this invention in hybridization methods (such as Southern blot analyses of genomic human DNAs), especially the most preferred DNAs listed herein above, without undue experimentation, it is  
20 possible to screen genomic or cDNA libraries to find other KGF-like DNAs which fall within the scope of this invention. Furthermore, by so using DNAs of this invention, genetic markers associated with the KGF gene, such as restriction  
25 fragment length polymorphisms (RFLPs), may be identified and associated with inherited clinical conditions involving this or other nearby genes.



This invention also includes modified forms of KGF DNAs. According to a chief embodiment of this aspect of the invention, such modified DNAs encode KGF-like proteins comprising segments of amino acid sequences of KGF and at least one other member of the FGF peptide family. Thus, for example, since there is no significant N-terminal homology between the secreted form of KGF and analogous positions in other FGF-related proteins, polypeptides with novel structural and functional properties may be created by grafting DNA segments encoding the distinct N-terminal segments of another polypeptide in the FGF family onto a KGF DNA segment in place of its usual NH<sub>2</sub>-terminal sequence.

The polypeptide chimeras produced by such modified DNAs are useful for determining whether the KGF NH<sub>2</sub>-terminal domain is sufficient to account for its unique target cell specificity. Studies on chimeras should also provide insights into which domains contribute the different effects of heparin on their biologic activities.

Indeed, the utility of this approach has already been confirmed by the successful engineering and expression of a chimeric molecule in which ~~about 40~~<sup>46</sup> amino acids from the NH<sub>2</sub>-terminus of the secreted form of KGF (beginning

with the amino terminal cys residue of the mature KGF form, numbered 32 in Fig <sup>7</sup>~~II-1~~, and ending at KGF residue 78, arg) is linked to <sup>116</sup>~~about 140~~ amino acids of the <sup>C</sup>~~CO<sub>2</sub>~~-terminal core of aFGF (beginning at residue 39, arg, and continuing to the C-terminal end of the aFGF coding sequence. This chimeric product has a target cell preference for keratinocytes, like KGF, but <sup>its activity is enhanced</sup>~~lacks susceptibility~~ to heparin, a characteristic which parallels that of aFGF rather than KGF. This novel KGF-like growth factor may have advantages in clinical applications where administration of an epithelial-specific growth factor is desirable in the presence of heparin, a commonly used anticoagulant. Further details of the construction of this chimeric molecule and the properties of the polypeptide are described in Experimental Section II.

Other DNAs of this invention include the following recombinant DNA molecules comprising a KGF cDNA and any of the following exemplary vector DNAs: a bacteriophage  $\lambda$  cloning vector ( $\lambda$ pCEV9); a DNA sequencing plasmid vector (a pUC variant); a bacterial expression vector (pKK233-2); or a mammalian expression vector (pMMT/neo). Such recombinant DNAs are exemplified by

constructs described in detail in the  
Experimental Sections.

Most preferred recombinant molecules  
include the following: molecules comprising the  
5 coding sequence for the secreted form of KGF and  
a bacterial expression vector (e.g., pKK233-2) or  
a cDNA encoding the entire primary translation  
product (including the <sup>N</sup>NH<sub>2</sub>-terminal signal  
peptide) and a mammalian expression vector  
10 (exemplified by pMMT) capable of expressing  
inserted DNAs in mammalian (e.g., NIH/3T3) cells.

Construction of recombinant DNAs  
containing KGF DNA and a bacterial expression  
vector is described in Experimental Section II.  
15 In brief, KGF cDNA was expressed to produce  
polypeptide in *E. coli* by placing its coding

sequence under control of the hybrid trk promoter  
<sup>Amman, E. and Brosius, J., 1985, Gene 40, 183</sup>  
B in the plasmid expression vector pKK233-2 (II-  
B 31).

20 Construction of recombinant DNAs  
comprising KGF DNA and a mammalian vector capable  
of expressing inserted DNAs in cultured human or  
animal cells, can be carried out by standard gene  
expression technology using methods well known in  
25 the art for expression of such a relatively  
simple polypeptide. One specific embodiment of a  
recombinant DNA of this aspect of the present

invention, involving the mammalian vector pMMT, is described further below in this section under recombinant cells of this invention.

DNAs and sense strand RNAs of this invention can be employed, in conjunction with protein production methods of this invention, to make large quantities of substantially pure KGF or KGF-like proteins. Substantially pure KGF protein thus produced can be employed, using well-known techniques, in diagnostic assays to determine the presence of receptors for this protein in various body fluids and tissue samples.

Accordingly, this invention also comprises a cell, preferably a bacterial or mammalian cell, transformed with a DNA of the invention, wherein the transforming DNA is capable of being expressed. In a preferred embodiment of this aspect of the invention, the cell transformed by the DNA of the invention produces KGF protein in a fully mitogenic form. Most preferably, these proteins will be of a secreted form (i.e., lacking an apparent signal sequence). These protein factors can be used for functional studies, and can be purified for additional biochemical and functional analyses, such as qualitative and quantitative receptor binding assays.

Recombinant *E. coli* cells have been constructed in a bacterial expression vector, pKK233-2, for production of KGF, as detailed in Experimental Section II. In summary, several  
5 recombinant bacterial clones were tested for protein production by the usual small scale methods. All recombinants tested synthesized a protein that was recognized by antibodies raised against an amino-terminal KGF peptide (see  
10 below). One recombinant was grown up in a one liter culture which produced recombinant KGF that efficiently stimulated thymidine incorporation into DNA of BALB/MK keratinocyte cells, but was only marginally active on NIH/3T3 fibroblasts.  
15 Half-maximal stimulation of the BALB/MK cells in the standard keratinocyte bioassay was achieved with a concentration of between 2 to 5 ng/ml, compared to a concentration of 10 to 15 ng/ml for KGF purified from M426 cells.

20 One liter of bacterial cells yielded approximately 50  $\mu$ g of Mono-S purified recombinant KGF. It will be apparent to those skilled in the art of gene expression that this initial yield can be improved substantially  
25 without undue experimentation by application of a variety known recombinant DNA technologies.

Recombinant mammalian (NIH/3T3 mouse) cells have also been constructed using the entire

KGF cDNA coding sequence (including the NH<sub>2</sub>-terminal signal peptide) and the vector pMMT/neo, which carries mouse metallothionine (MMT) promoter and the selective marker gene for neomycin resistance. The cells are being evaluated for KGF production, particularly for secretion of the mature form (lacking signal peptide) produced by human fibroblasts, using bioassays of the present invention. This same vector and host cell combination has been used successfully to express several other similar recombinant polypeptides, including high levels of Platelet-Derived Growth Factor (PDGF) A and B chains (11-32). Accordingly, it will be recognized by those skilled in the art that high yields of recombinant KGF can be achieved in this manner, using the aforementioned recombinant DNAs and transformed cells of this invention.

Ultimately, large-scale production can be used to enable clinical testing in conditions requiring specific stimulation of epithelial cell growth. Materials and methods for preparing pharmaceutical compositions for administration of polypeptides topically (to skin or to the cornea of the eye, for example) or systemically are well known in the art and can be adapted readily for

administration of KGF and KGF-like peptides without undue experimentation.

This invention also comprises novel antibodies made against a peptide encoded by a DNA segment of the invention. This embodiment of the invention is exemplified by several kinds of antibodies which recognize KGF. These have been prepared using standard methodologies well known in the art of experimental immunology, as outlined in Experimental Section II. These antibodies include: monoclonal antibodies raised in mice against intact, purified protein from human fibroblasts; polyclonal antibodies raised in rabbits against synthetic peptides with sequences based on amino acid sequences predicted from the KGF cDNA sequence [exemplified by a peptide with the sequence of KGF residues 32-45, <sup>45 plus an hist. its C-terminus</sup> namely, NDMTPEQMATNVR (using standard one-letter code for amino acid sequences; see Fig. <sup>1</sup>II-1)]; polyclonal antibodies raised in rabbits against both naturally secreted KGF from human fibroblasts and recombinant KGF produced in *E. coli* (see above).

All tested antibodies recognize the recombinant as well as the naturally occurring KGF, either in a solid-phase (ELISA) assay and/or in a Western blot. Some exemplary antibodies, which are preferred antibodies of this invention,

appear to neutralize mitogenic activity of KGF in the BALB/MK bioassay.

5       Fragments of antibodies of this invention, such as Fab or F(ab)' fragments, which retain antigen binding activity and can be prepared by methods well known in the art, also fall within the scope of the present invention. Further, this invention comprises pharmaceutical compositions of the antibodies of this invention, 10 or active fragments thereof, which can be prepared using materials and methods for preparing pharmaceutical compositions for administration of polypeptides that are well known in the art and can be adapted readily for 15 administration of KGF and KGF-like peptides without undue experimentation.

      These antibodies, and active fragments thereof, can be used, for example, for detection of KGF in bioassays or for purification of the 20 protein factors. They may also be used in approaches well known in the art, for isolation of the receptor for KGF, which, as described in Experimental Section II, appears to be distinct from those of all other known growth factors.

25       Those preferred antibodies, and fragments and pharmaceutical compositions thereof, which neutralize mitogenic activity of KGF for epithelial cells, as indicated by the



BALB/MK assay, for instance, may be used in the treatment of clinical conditions characterized by excessive epithelial cell growth, including dysplasia and neoplasia (e.g., psoriasis, or malignant or benign epithelial tumors).

This invention further comprises novel bioassay methods for detecting the expression of genes related to DNAs of the invention. In some exemplary embodiments, DNAs of this invention were used as probes to determine steady state levels of related mRNAs. Methods for these bioassays of the invention, using KGF DNAs, and standard Northern blotting techniques, are described in detail in Experimental Section II.

One skilled in the art will recognize that, without undue experimentation, such methods may be readily applied to analysis of gene expression for KGF-like proteins, either in isolated cells or various tissues. Such bioassays may be useful, for example, for identification of various classes of tumor cells or genetic defects in the epithelial growth processes.

Without further elaboration, it is believed that one of ordinary skill in the art, using the preceding description, and following the methods of the Experimental Sections below, can utilize the present invention to its fullest

extent. The material disclosed in the  
Experimental Sections, unless otherwise  
indicated, is disclosed for illustrative purposes  
and therefore should not be construed as being  
5      limitive in any way of the appended claims.

## EXPERIMENTAL SECTION I

### IDENTIFICATION AND CHARACTERIZATION OF A NOVEL GROWTH FACTOR SPECIFIC FOR EPITHELIAL CELLS

This section describes experimental work  
5 leading to identification of a growth factor  
specific for epithelial cells in conditioned  
medium of a human embryonic lung fibroblast cell  
line. The factor, provisionally termed  
keratinocyte growth factor (KGF) because of its  
10 predominant activity on this cell type, was  
purified to homogeneity by a combination of  
ultrafiltration, heparin-Sepharose affinity  
chromatography and hydrophobic chromatography on  
a C<sub>4</sub> reversed-phase HPLC column, according to  
15 methods of this invention. KGF was found to be  
both acid and heat labile, and consisted of a  
single polypeptide chain with an apparent  
molecular weight of approximately 28,000 daltons.  
Purified KGF was a potent mitogen for epithelial  
20 cells, capable of stimulating DNA synthesis in  
quiescent BALB/MK epidermal keratinocytes by more  
than 500-fold with activity detectable at 0.1 nM  
and maximal at 1.0 nM. Lack of mitogenic  
activity on either fibroblasts or endothelial  
25 cells indicated that KGF possessed a target cell  
specificity distinct from any previously  
characterized growth factor. Microsequencing

revealed an amino-terminal sequence containing no significant homology to any known protein. The release of this novel growth factor by human embryonic fibroblasts indicates that KGF plays a role in mesenchymal stimulation of normal epithelial cell proliferation.

#### METHODS AND MATERIALS

Preparation of Conditioned Media. An early passage of M426 human embryonic fibroblasts (<sup>auth</sup>~~1-8~~) was plated onto 175 cm<sup>2</sup> T-flasks and grown to confluence over 10-14 days in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% calf serum (GIBCO). Once confluent, the monolayers were cycled weekly from serum-containing to serum-free medium, the latter consisting of DMEM alone. The cells were washed twice with 5 ml of phosphate buffered saline prior to addition of 20 ml of DMEM. After 72 hrs, culture fluids were collected and replaced with 35 ml of serum-containing medium. The conditioned medium was stored at -70°C until further use.

Ultrafiltration. Approximately ten liters of conditioned medium were thawed, prefiltered through a 0.50 micron filter (Millipore HAWP 142 50) and concentrated to 200

ml using the Pellicon cassette system (Millipore XX42 00K 60) and a cassette having a 10 kDa molecular weight cutoff (Millipore PTGC 000 05). After concentration, the sample was subjected to two successive rounds of dilution with one liter of 20 mM Tris-HCl, pH 7.5/0.3M NaCl, each followed by another step of ultrafiltration with the Pellicon system. Activity recovered in the retentate was either immediately applied to heparin-Sepharose resin or stored at -70°C.

Heparin-Sepharose Affinity Chromatography (HSAC). The retentate from ultrafiltration was loaded onto heparin-Sepharose resin (Pharmacia) which had been equilibrated in 20 mM Tris-HCl, pH 7.5/0.3 M NaCl. The resin was washed extensively until the optical density had returned to baseline and then subjected to a linear-step gradient of increasing NaCl concentration. After removing aliquots from the fractions for the thymidine incorporation bioassay, selected fractions were concentrated ten- to twenty-fold with a Centricon-10 microconcentrator (Amicon) and stored at -70°C.

Reversed-Phase HPLC (RP-HPLC). Active fractions (0.6 M NaCl pool) from the HSAC were thawed, pooled and further concentrated with the Centricon-10 to a final volume of  $\leq 200 \mu\text{l}$ . The sample was loaded onto a Vydac C<sub>4</sub> HPLC column (The

Separations Group, Hesperia, CA) which had been equilibrated in 0.1% trifluoroacetic acid (TFA, Fluka)/20% acetonitrile (Baker, HPLC grade) and eluted with a linear gradient of increasing acetonitrile. Aliquots for the bioassay were immediately diluted in a 10-fold excess of 50 µg/ml BSA (Fraction V, Sigma)/20 mM Tris-HCl, pH 7.5. The remainder of the sample was dried in a Speed-Vac (Savant) in preparation for structural analysis.

Molecular Sieve HPLC. Approximately 50 µl of the twice concentrated heparin-Sepharose fractions were loaded onto a TSK-G3000SW Glas-Pac Column (LKB) which had been equilibrated in 20 mM Tris-HCl, pH 6.8/0.5M NaCl. The sample was eluted in this buffer at a flow rate of 0.4 ml/min. After removing aliquots for the bioassay, the fractions were stored at -70°C.

NaDodSO<sub>4</sub>-Polyacrylamide Gel

Electrophoresis (NaDodSO<sub>4</sub>/PAGE). Polyacrylamide gels were prepared with NaDodSO<sub>4</sub> according to the procedure of Laemmli (1970, *Nature* 227, 680-685) <sup>Laemmli U.K., 1970, Nature 227, 680-685</sup> (1-9). Samples were boiled for 3 min in the presence of 2.5%

2-mercaptoethanol (vol/vol). The gels were fixed and stained with silver <sup>Merril, C.R., et al., 1981, Science 211, 1437-1438</sup> (1-10) using the reagents and protocol from BioRad. Molecular weight markers were from Pharmacia.

DNA Synthesis Stimulation. Ninety-six

well microtiter plates (Falcon No. 3596) were  
precoated with human fibronectin (Collaborative  
Research) at 1  $\mu\text{g}/\text{cm}^2$  prior to seeding with  
5 BALB/MK cells. Once confluent, the cells were  
maintained for 24-72 hr in serum-free medium  
containing 5  $\mu\text{g}/\text{ml}$  transferrin (Collaborative  
Research) and 30 nM  $\text{Na}_2\text{SeO}_3$  (Baker).

Incorporation of  $^3\text{H}$ -thymidine (5  $\mu\text{Ci}/\text{ml}$  final  
10 concentration, NEN) into DNA was measured during  
a 6 hr period beginning at 16 hrs following  
addition of samples. The assay was terminated by  
washing the cells once with ice cold phosphate-  
buffered saline and twice with 5% trichloroacetic  
15 acid. The precipitate was redissolved in 0.25 M  
NaOH, transferred into liquid scintillation fluid  
(Biofluor, NEN) and counted.

Stimulation of DNA synthesis was  
monitored as described above for BALB/MK cells on

20 a variety of other cell lines. NIH/3T3  
*Jainchill, J. L., Garonson, S. A. and Todaro, H. G., 1969, J. Virol. 4, 549-553*  
B fibroblasts ( $\text{F-11}$ ) were available from the

National Institutes of Health, while CCL208  
B *Caputo, J. L., Hay, R. J. and Williams, C. D., 1979, In Vitro 15, 222-223*  
Rhesus monkey bronchial epithelial cells ( $\text{F-12}$ )

were obtained from the American Type Culture

25 Collection. The B5/589 human mammary epithelial  
*Stampfer, M. R. and Bartley, J. C., 1985, Proc. Natl. Acad. Sci. USA 82, 2394-2398*  
B cell line, prepared as described in ( $\text{F-13}$ ), was

B obtained from Martha Stampfer (*Lawrence Berkeley*  
*Laboratory* ~~University of~~  
B ~~California~~, Berkeley). The mammary cells were

grown in RPMI 1640 supplemented with 10% fetal calf serum and 4 ng/ml EGF. When maintained in serum-free conditions, the basal medium was DMEM.

Primary cultures of human saphenous vein

5 endothelial cells were prepared and maintained as  
B *Shenglin, J.B., Fanchild, K.D., Albus, R.A., Crueses, D.F. and Lick, W.M., 1986, J. Surgical Res. 41, 483-472*  
described elsewhere (1-14). Epidermal growth

factor and insulin were from Collaborative

Research. Acidic FGF and bFGF were obtained from California Biotechnology, Inc. Recombinant TGF $\alpha$

10 was obtained from Genentech, Inc. Media and serum were either from GIBCO, Biofluids, Inc. or the NIH media unit.

Proliferation Assay. Thirty-five mm culture dishes were precoated sequentially with  
15 poly-D-lysine (20  $\mu\text{g}/\text{cm}^2$ ) (Sigma) and human fibronectin, and then seeded with approximately  $2.5 \times 10^4$  BALB/MK cells. The basic medium was a 1:1 mixture of Eagle's low  $\text{Ca}^{2+}$  minimal essential medium and Ham's F-12 medium, supplemented with 5  
20  $\mu\text{g}/\text{ml}$  transferrin, 30 nM  $\text{Na}_2\text{SeO}_3$  and 0.2 mM ethanolamine (Sigma). Medium was changed every 2 or 3 days. After 10 days, the cells were fixed in formalin (Fisher Scientific Co.) and stained with Giemsa (Fisher Scientific Co.).

25 Protein microsequencing. Approximately 4  $\mu\text{g}$  (.150 pmol) of protein from the active fractions of the  $\text{C}_4$  column were redissolved in 50% TFA and loaded onto an Applied Biosystems gas-



phase protein sequenator. Twenty rounds of Edman degradation were carried out and identifications of amino acid derivatives were made with an automated on-line HPLC (Model 120A, Applied Biosystems).

## RESULTS

### Growth Factor Detection and Isolation.

Preliminary screening of conditioned media from various cell lines indicated that media from some fibroblast lines contained mitogenic activities detectable on both BALB/MK and NIH/3T3 cells.

Whereas boiling destroyed the activity on BALB/MK, mitogenic activity on NIH/3T3 remained

intact. Based on the known heat stability of EGF

*Cohen, S., 1962, J. Biol. Chem. 237, 1555-1562; Delarosa, J. E. and Tostero, H. L., 1978, Proc. Natl. Acad. Sci. USA 75, 4201-4205*

15 (I-15) and TGF $\alpha$  (I-16), it was reasoned that the

BALB/MK mitogenic activity might be due to an agent different from these known epithelial growth factors.

M426, a human embryonic lung fibroblast line, was selected as the most productive source of this activity for purification of the putative growth factor(s). Ultrafiltration with the Pellicon system provided a convenient way of reducing the sample volume to a suitable level for subsequent chromatography. Various combinations of sieving, ion exchange and



Final purification was achieved by RP-HPLC with a C<sub>4</sub> Vydac column, a preparative method suitable for amino acid sequence analysis. While the yield of activity from the C<sub>4</sub> step was usually only a few percent, this loss could be attributed to the solvents employed. In other experiments, exposure to 0.1% TFA/50% acetonitrile for 1 hr at room temperature reduced the mitogenic activity of the preparation by 98%. Nonetheless, as shown in Fig. <sup>2A</sup>~~1~~<sub>2</sub>, a single peak of BALB/MK stimulatory activity was obtained, coinciding with a distinct peak in the optical density profile. The peak fractions produced a single band upon NaDodSO<sub>4</sub>/PAGE and silver staining of the gel (Fig. ~~1~~<sup>2</sup>B), and the relative mitogenic activity of each tested fraction (Fig. ~~1~~<sup>2</sup>C) correlated well with the intensity of the bands across the activity profile.

An alternative purification step to the HPLC technique described above, using sieving chromatography with a TSK G3000SW GlasPac column run in aqueous solution near physiologic pH, resulted in a major peak of activity in the BALB/MK bioassay (Fig. ~~1~~<sup>3</sup>). This preparation was almost as pure as the one obtained from RP-HPLC as judged by silver-stained NaDodSO<sub>4</sub>/PAGE (data not shown) but provided a far better recovery of activity (Table ~~1~~<sup>1</sup>). The TSK-

purified material was used routinely for biological studies as it had a higher specific activity.

In both types of purified preparations (i.e., purified by HPLC or molecular sieving), the profile of mitogenic activity was associated with a distinct band on NaDodSO<sub>4</sub>/PAGE which appeared to be indistinguishable in the two preparations.

Physical and Biological Characterization of the Growth Factor. The purified factor had an estimated molecular weight of about 28 kDa based on NaDodSO<sub>4</sub>/PAGE under reducing (Fig. <sup>2A-2C</sup>~~1-2~~) and non-reducing conditions (data not shown). This value was in good agreement with its elution position on two different sizing columns run in solvents expected to maintain native conformation (TSK-G3000-SW, Fig. ~~1-3~~<sup>2</sup>, and superose-12, data not shown). From these data, the mitogen appears to consist of a single polypeptide chain with a molecular weight of 25-30 kDa.

The heat and acid lability of the mitogenic activity were demonstrated using the BALB/MK mitogenesis bioassay. While activity was unaffected by a 10 min incubation at 50°C, it was reduced by 68% after 10 min at 60°C and was undetectable after 3 min at 100°C. Exposure to 0.5M acetic acid for 60 min at room temperature

resulted in a decline in activity to 14% of the control. In comparison, the mitogenic activity of the known growth factor, EGF, was not diminished by any of these treatments.

P 5           The dose response curve for the purified growth factor depicted in Fig. 1-4 illustrates that as little as 0.1 nM led to a detectable stimulation of DNA synthesis. Thus, the activity range was comparable to that of the other growth factors analyzed to date. A linear relationship was observed in the concentration range 0.1 - 1.0 nM with maximal stimulation of 600 fold observed at 1.0 nM. The novel factor consistently induced a higher level of maximal thymidine incorporation than EGF, aFGF, or bFGF in the BALB/MK keratinocytes (Fig. 1-4).

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P 20           The distinctive target cell specificity of this factor was demonstrated by comparing its activities on a variety of cell types with those of other growth factors known to possess epithelial cell mitogenic activity. As shown in Table 1-2, the newly isolated factor exhibited a strong mitogenic effect on BALB/MK but also induced demonstrable incorporation of thymidine into DNA of the other epithelial cells tested. In striking contrast, the factor had no detectable mitogenic effects on mouse (or human,

25

data not shown) fibroblasts or human saphenous vein endothelial cells.

By comparison, none of the other known growth factors appeared to preferentially stimulate keratinocytes. TGF $\alpha$  and EGF showed potent activity on fibroblasts, while the FGFs were mitogenic for endothelial cells as well as fibroblasts (Table 1-2). Because of its specificity of epithelial cells and the sensitivity of keratinocytes in particular, the novel mitogen was provisionally designated as keratinocyte growth factor (KGF).

To establish that KGF not only would stimulate DNA synthesis but would also support sustained cell growth, the ability of BALB/MK cells to grow in a fully-defined, serum-free medium supplemented with this growth factor was assessed. As shown in Fig. 1-5, KGF served as an excellent substitute for EGF but not insulin (or insulin-like growth factor I) in this chemically defined medium. Thus, KGF appears to act through the major signalling pathway shared by EGF, aFGF and bFGF for proliferation of BALB/MK cells.

Microsequencing Reveals a Unique N-terminal Amino Acid Sequence of KGF. To further characterize the growth factor, approximately 150 pmol of C<sub>4</sub>-purified material were subjected to amino acid sequence analysis. A single sequence

was detected with unambiguous assignments made for cycles 2-13, as follows: X-Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala-Thr-Asn-Val. High background noise precluded an assignment for the first position which is, therefore, indicated by an X.

B A computer search using the FASTP program (1-24) revealed that the N-terminal amino

acid sequence of KGF showed no significant homology to any protein in the National Biomedical Research Foundation data bank, thus supporting the novelty of this epithelial growth factor.

#### DISCUSSION

The studies described in this Experimental Section identified a human growth factor which has a unique specificity for epithelial cells. By employing ultrafiltration, HSAC and RP-HPLC or TSK sieving chromatography according to the present invention, a quantity sufficient to permit detailed characterization of the physical and biological properties of this molecule was isolated.

A single silver-stained band corresponding to a molecular weight of about 28,000 daltons was detected in the active fractions from RP-HPLC, and the intensity of the

band was proportional to the level of mitogenic activity in these fractions. A band indistinguishable from that obtained by RP-HPLC was seen in the active fractions from TSK chromatography. The purified protein stimulated DNA synthesis in epithelial cells at sub-nanomolar concentrations, but failed to induce any thymidine incorporation in fibroblasts or endothelial cells at comparable or higher concentrations (up to 5 nM). This distinctive target cell specificity combined with the single novel N-terminal amino acid sequence determined from the purified molecule lead to the conclusion that KGF represents a new growth factor.

In a chemically defined medium the purified factor was able to complement the insulin-like growth factor I/insulin growth requirement of BALB/MK cells and therefore must act through a signal transduction pathway shared with EGF, TGF $\alpha$  and the FGFs. Moreover, the new factor was more potent than any of the known epithelial cell mitogens in stimulating thymidine incorporation in BALB/MK cells. Preliminary evidence indicates that this factor is also capable of supporting proliferation of secondary cultures of human keratinocytes (data not shown).

Handling and storage of KGF were problematical during its purification. Besides



its inherent lability to acid and heat, it was unstable to lyophilization or dialysis. After HSAC, complete loss of activity occurred within 24 hr despite the use of carrier proteins, heparin, protease inhibitors, siliconized tubes or storage at either 4° or -20°C. Only concentrating the sample at this stage could preserve its activity.

Furthermore, in order to transfer the dried, purified factor it was necessary to utilize either strong acid or detergent, consistent with an adsorptive tendency or insolubility. Thus, for preservation of activity, the purified factor was maintained in solution at high concentrations at -70°C where it remained stable for several months.

The ability of KGF to bind heparin may signify a fundamental property of this factor that has a bearing on its function *in vivo*. Growth factors with heparin-binding properties include aFGF (I-20, I-22) bFGF (I-19, I-22) (Roberts R., et al., 1988, Nature 332, 376-378) granulocyte/macrophage colony stimulating factor (Roberts R., et al., 1988, Nature 332, 376-378) and interleukin 3 (I-25). Each of these is produced by stromal cells (I-25, I-27). Such factors appear to be deposited in the extracellular matrix, or on proteoglycans coating the stromal cell surface (I-25, I-28). It has been postulated that their storage, release and

contact with specific target cells are regulated  
by this interaction (~~I-25~~, ~~I-28~~). While  
mesenchymal-derived effectors of epithelial cell  
proliferation have also been described (~~I-29~~ ~~I-31~~ ~~I-32~~ ~~I-33~~ ~~I-34~~ ~~I-35~~ ~~I-36~~ ~~I-37~~ ~~I-38~~ ~~I-39~~ ~~I-40~~ ~~I-41~~ ~~I-42~~ ~~I-43~~ ~~I-44~~ ~~I-45~~ ~~I-46~~ ~~I-47~~ ~~I-48~~ ~~I-49~~ ~~I-50~~ ~~I-51~~ ~~I-52~~ ~~I-53~~ ~~I-54~~ ~~I-55~~ ~~I-56~~ ~~I-57~~ ~~I-58~~ ~~I-59~~ ~~I-60~~ ~~I-61~~ ~~I-62~~ ~~I-63~~ ~~I-64~~ ~~I-65~~ ~~I-66~~ ~~I-67~~ ~~I-68~~ ~~I-69~~ ~~I-70~~ ~~I-71~~ ~~I-72~~ ~~I-73~~ ~~I-74~~ ~~I-75~~ ~~I-76~~ ~~I-77~~ ~~I-78~~ ~~I-79~~ ~~I-80~~ ~~I-81~~ ~~I-82~~ ~~I-83~~ ~~I-84~~ ~~I-85~~ ~~I-86~~ ~~I-87~~ ~~I-88~~ ~~I-89~~ ~~I-90~~ ~~I-91~~ ~~I-92~~ ~~I-93~~ ~~I-94~~ ~~I-95~~ ~~I-96~~ ~~I-97~~ ~~I-98~~ ~~I-99~~ ~~I-100~~ ~~I-101~~ ~~I-102~~ ~~I-103~~ ~~I-104~~ ~~I-105~~ ~~I-106~~ ~~I-107~~ ~~I-108~~ ~~I-109~~ ~~I-110~~ ~~I-111~~ ~~I-112~~ ~~I-113~~ ~~I-114~~ ~~I-115~~ ~~I-116~~ ~~I-117~~ ~~I-118~~ ~~I-119~~ ~~I-120~~ ~~I-121~~ ~~I-122~~ ~~I-123~~ ~~I-124~~ ~~I-125~~ ~~I-126~~ ~~I-127~~ ~~I-128~~ ~~I-129~~ ~~I-130~~ ~~I-131~~ ~~I-132~~ 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## EXPERIMENTAL SECTION II

### CDNA SEQUENCE OF A NOVEL EPITHELIAL CELL SPECIFIC GROWTH FACTOR DEFINES A NEW MEMBER OF THE FGF FAMILY

5 Work in the previous Experimental  
Section I identified and purified a novel  
heparin-binding growth factor, designated  
keratinocyte growth factor (KGF), which is  
particularly active on keratinocytes and appears  
10 to be specific for epithelial cells. This second  
Experimental Section describes the isolation and  
characterization of cDNA clones encoding KGF,  
using synthetic oligonucleotides, based upon the  
experimentally determined <sup>N</sup>NH<sub>2</sub>-terminal amino acid  
15 sequence, as hybridization probes. Nucleotide  
sequence analysis identified a 582-bp open  
reading frame which would code for a 194-amino  
acid polypeptide that is between 41% and 33%  
identical to the heparin-binding acidic and basic  
20 fibroblast growth factors (FGFs), and the related  
products of the <sup>FGF-S</sup>~~hst~~<sub>A</sub> and *int-2* oncogenes. The KGF  
F β gene RNA transcript is expressed in normal  
fibroblasts of both embryonic and adult origin,  
but not in epithelial, endothelial or glial  
25 cells. Thus, KGF appears to be normally  
expressed by the mesenchyme, indicating a role in  
the regulation of epithelial cell proliferation.

## MATERIALS AND METHODS

### Isolation of cDNA clones. The

purification and N-terminal sequencing of KGF has

been previously described (see Experimental

Rubin, J.S., Asada, H., Furch, P.W., Taylor, W.G., Rudikoff, S. and Carson, S.A., 1989, *Proc. Natl. Acad. Sci. USA* (in press), February, 1989  
5  $\beta$  Section I, above and II-3). Pools (50 pmole) of

deoxyoligonucleotides described under Results

were 5' end-labelled using 83 pmole of  $\gamma$ - $^{32}$ P-ATP

(3000 Ci/mmole, Amersham) and 10 units of T4

polynucleotide kinase. The recombinant phage

10 carrying cDNA clones were replica plated onto

nitrocellulose filters and hybridized with

$^{32}$ P-labelled deoxyoligonucleotides in 20%

formamide, 10% dextran sulphate, 10 mM Tris-HCl

(pH 7.5), 8 x SSC, 5x Denhardt's and 50  $\mu$ g/ml

15 denatured salmon sperm DNA, overnight at 42°C.

Filters were washed in 0.5 x SSC, 0.1% SDS at

50°C and exposed to Kodak X-omat AR film.

### DNA sequencing. The nucleotide sequence

of the KGF cDNA was determined by the dideoxy

Sanger, F., Nicklen, S. and Coulson, A.R., 1977, *Proc. Natl. Acad. Sci. USA* 74, 5463-5467  
20 chain termination method (II-26), of overlapping

restriction fragments, subcloned into pUC vectors

Yanisch-Perron, C., Vieira, J. and Messing, J., 1985, *Gene* 33, 103-119  
B (II-27)

### Construction of a bacterial expression

vector for KGF cDNA. KGF cDNA encoding the

25 mature, secreted form of the polypeptide was

placed under control of the hybrid trk promoter

Amman, E. and Brosius, J., 1985, *Here*

β in the plasmid expression vector pKK233-2 (II-  
40, 193 31), as follows. To accomplish this, a specific  
length of KGF cDNA that contained the information  
to code for the mature KGF molecule (i.e.,  
5 without its signal peptide) was amplified using  
the polymerase chain reaction (PCR) technique  
(II-32). The fragment was directionally inserted  
between two sites in the vector, namely the *Nco*I  
site, made blunt ended by *S*1 nuclease digestion,  
10 and the *Hind*III site, using standard recombinant  
DNA methodology. The ends of the KGF cDNA  
produced by the PCR method were as follows: the  
5' end was blunt and began with an ATG codon,  
followed by the codon TGC for cys residue, number  
15 33, which is the amino terminal residue of the  
mature form of KGF (see Fig. II-1), and then the  
entire KGF coding sequence. The stop codon, TAA,  
and the four bases immediately following, TTGC,  
were also included on the 3' end of the cDNA.  
20 The primer used in the PCR method to direct DNA  
synthesis to the desired position on the 3' end  
of the cDNA included a *Hind*III site for insertion  
of the amplified cDNA into the vector DNA.

Production of antibodies against KGF and

25 KGF-related peptides. Monoclonal antibodies were  
raised in mice against intact, purified protein  
from human fibroblasts using 5 or more



subcutaneous injections. Test bleeds were  
screened with a solid-phase (ELISA) assay using  
F ~~the conditioned medium of human fibroblast~~ human epithelial  
B highly purified KGF from human epithelial cells  
as antigen. Hybridomas were prepared by routine  
B 5 methods and <sup>supernatants</sup> ~~supernatants~~ were screened with the  
ELISA assay to detect KGF-reactive antibodies.  
Positive clones were serially subcloned by the  
usual methods, and selected subclones were grown  
as ascites tumors in mice for production of large  
10 amounts of antibodies. Antibodies were purified  
from ascites fluids employing standard techniques  
(e.g., hydroxyapatite or immunoaffinity resins).

Polyclonal antibodies against a  
synthetic peptide were raised in rabbits by  
15 standard methods, as follows. The peptides were  
made by solid phase technology and coupled to  
thyroglobulin by reaction with glutaraldehyde.  
Serial subcutaneous injections were made and test  
B ~~bleeds~~ <sup>bleeds</sup> were screened by ELISA as well as other  
20 techniques, including Western blot analysis and  
mitogenesis bioassay. IgG immunoglobulins were  
isolated by affinity chromatography using  
immobilized protein G.

Polyclonal antibodies were raised in  
25 rabbits against both naturally secreted KGF from  
human fibroblasts and recombinant KGF produced in  
*E. coli* (see next section), using the following  
protocol:

i) Initial injection and first boost were administered in the inguinal lymph nodes;

ii) subsequent boosts were made intramuscularly.

5

Screening of test bleeds included ELISA as well as Western blot analysis, and mitogenesis bioassay, and IgG was purified as for antibodies against synthetic peptides, above.

## RESULTS

Isolation of cDNA clones encoding the novel growth factor. To search for cDNA clones corresponding to the KGF coding sequence, two  
5 pools of oligonucleotides with lengths of 26 bases were generated based upon a nine-amino acid sequence, Asn-Asp-Met-Thr-Pro-Glu-Gln-Met-Ala, as determined by microsequencing of purified KGF (see Experimental Section I, above and ~~reference~~  
10 ~~II-3~~). One oligonucleotide pool contained a <sup>F13</sup> mixture of all 256 possible coding sequences for the nine amino acids, while the other contained inosine residues at the degenerate third position of the codons for Thr and Pro.

15 This latter design reduced the number of possible coding sequences in the pool to 16.

Inosine in a tRNA anticodon can form hydrogen  
Crick, F.H.C., 1966, J. Mol. Biol. 19, 548-555  
bonds with A, C or U (~~II-4~~), and oligonucleotides  
that contain deoxyinosine have been shown to

20 hybridize efficiently with the corresponding cDNA  
Ohtsuka, E., Matsuki, M., Ikebara, M., Takashi, Y. and Matsubara, K., 1985, J. Biol. Chem. 260, 2605-2608  
B (~~II-5~~).

A cDNA library was constructed in a cDNA  
Niki, T., Matsui, T., Heidaran, M. and Aaronson, S.A., unpublished observations  
B cloning vector, pCEV9 (~~II-6~~) using

polyadenylated RNA extracted from the human  
Aaronson, S.A. and Todaro, G.J. 1968, Virology 36, 254-261  
B 25 embryonic lung fibroblast cell line M426 (~~II-7~~),  
the initial source of the growth factor.

Screening of the library ( $9 \times 10^5$  plaques) with

the <sup>32</sup>P-labelled 26-mer oligonucleotides identified 88 plaques which hybridized to both pools of oligonucleotide probes.

5     Characterization and sequencing of  
      selected cDNA clones. Of 10 plaque-purified clones that were analyzed, one, designated clone 49, had a cDNA insert of 3.5 kb, while the rest had inserts ranging from 1.8 kb to 2.1 kb.

10     Analysis of the smaller clones revealed several common restriction sites. Sequencing of a representative smaller clone, designated clone 32, along with clone 49, demonstrated that they  
F     β were overlapping cDNAs (Fig. ~~II-1A~~<sup>6</sup><sub>1A</sub>). Whereas  
      β ~~clone 49 was primed from the poly(A) tail of the~~  
15 β ~~message, clone 32 arose during the construction~~  
      β ~~of the library by hybridization of the oligo (dT)~~  
      β ~~primer to an A-rich sequence in the 3' noncoding~~  
      β ~~region of the KGF mRNA.~~

20     Description of the sequence encoding the  
      KGF polypeptide. Alignment of the two cDNAs (clones 32 and 49) established a continuous sequence of 3.85 kb containing the complete KGF coding sequence (Fig. ~~II-1B~~<sup>7</sup><sub>1B</sub>). An ATG likely to  
F     be an initiation codon was located at nucleotide  
25     position 446, establishing a 582-base pair open reading frame that ended at a TAA termination codon at position 1030. This open reading frame

would encode a 194-amino acid polypeptide with a calculated molecular weight of 22,512 daltons.

The sequence flanking the ATG codon did not conform to the proposed GCC(G/A)CCATGG

5 consensus for optimal initiation by eukaryotic  
Koyok, M., 1987, Nucl. Acids Res. 13, 8125-8148  
B ribosomes (~~II-8~~), however, there was an A three  
nucleotides upstream of the ATG codon. An A at  
this position is the most highly conserved  
nucleotide in the consensus. This ATG codon was  
10 preceded 85 nucleotides upstream by a TGA stop  
codon in the same reading frame.

A 19-amino acid sequence that was homologous to the experimentally determined  
N  
NH<sub>2</sub>-terminus of purified KGF began 32 amino acids  
A  
15 downstream of the proposed initiation codon.  
There was complete agreement between the  
predicted and experimentally determined amino  
acid sequences, where unambiguous assignments  
could be made.

20 To search for homology between KGF and  
any known protein, a computer search of the  
National Biomedical Research Foundation data base  
using the FASTP program of Lipman and Pearson was  
Lipman D.J. and Pearson, R.W., 1985, Science 227, 1435-1443  
B conducted (~~II-9~~). By this approach, a striking  
25 degree of relatedness between the predicted  
primary structure of KGF and those of acidic and

1- B basic FGF, as well as the related ~~FGF-5~~ <sup>hst</sup> and  
int-2-encoded proteins was revealed.

Expression of mRNA transcripts of the  
KGF gene in human cells. In preliminary attempts  
5 to examine expression of KGF mRNA in human cells,  
a probe spanning the majority of the KGF coding  
sequence (Probe A, Figure ~~II-1A~~ <sup>6</sup><sub>A</sub>) detected a  
single 2.4 kb transcript by Northern blot  
analysis of total M426 RNA (Figure ~~II-1E~~ <sup>8</sup><sub>A</sub>). This  
10 was considerably shorter than the length of the  
composite cDNA sequence, 3.85 kb.

However, on screening poly(A)-selected  
M426 RNA, an additional transcript of  
approximately 5 kb was detected. Furthermore, a  
15 probe derived from the untranslated region of  
clone 49, 3' to the end of clone 32 (Probe B,  
Figure ~~II-1A~~ <sup>6</sup><sub>A</sub>), hybridized only to the larger  
message (Figure ~~II-1E~~ <sup>8</sup><sub>A</sub>). Thus, it appears that  
the KGF gene is transcribed as to alternate RNAs.

20 Two other members of the FGF gene family, bFGF  
Abraham J.A., et al., 1986, Science 233, 545-548 Ransaw, A.L. and Martin, A.R., 1988, EMBO J. 1, 2035-2041  
B (II-29) and int-2 (~~II-30~~ <sup>10</sup><sub>A</sub>), also express multiple  
RNAs, the significance of which remains to be  
determined.

To investigate the normal functional  
25 role of KGF, the expression of its transcript in  
a variety of human cell lines and tissues was  
examined. As shown in Figure ~~II-3~~ <sup>10</sup><sub>A</sub>, the

predominant 2.4 kb KGF transcript was detected in each of several stromal fibroblast lines derived from epithelial tissues of embryonic, neonatal and adult sources, but not from epithelial cell lines of normal origin. The transcript was also detected in RNA extracted from normal adult kidneys and organs of the gastrointestinal tract, but not from lung or brain. The striking specificity of KGF RNA expression in stromal cells from epithelial tissues indicated that this factor plays a normal role in mesenchymal stimulation of epithelial cell growth.

For comparison, the mRNAs of other growth factors with known activity on epithelial cells were also analyzed in the same tissues as listed above. Among the epithelial and stromal cell lines analyzed, there was no consistent pattern of expression of aFGF or bFGF transcripts (Fig. ~~II-3~~<sup>10</sup>). The EGF transcript was not expressed <sup>in</sup> any of the same cell lines, and was only observed in kidney, among the various tissues. Finally, the TGF $\alpha$  message was not detected in any of the stromal fibroblast lines and was expressed at varying levels in each of the epithelial cell lines. It was also detected at low levels in kidney among the tissues examined (Fig. ~~II-3~~<sup>10</sup>).

Inhibition of KGF mitogenic activity by  
heparin. Heparin has been shown to substantially  
increase the mitogenic activity of aFGF for a  
variety of target cells in culture, and to  
5 stabilize it from heat inactivation (~~II-21, II-~~  
~~22~~). Despite binding tightly to bFGF, heparin<sup>F14</sup>  
had minimal effects on its mitogenic activity  
(~~II-22~~). In view of the relatedness of KGF to  
the FGFs, the effect of heparin on KGF mitogenic<sup>F15</sup>  
10 activity was examined. As shown in Table II-1,  
thymidine incorporation by BALB/MK cells in  
response to KGF was inhibited 16 fold when  
heparin was included in the culture medium. In  
contrast, the activities of both aFGF and bFGF  
15 were increased by the same treatment.

Production of anti-KGF antibodies.

Several kinds of antibodies which recognize KGF  
or KGF-like polypeptides have been prepared using  
standard methodologies well known in the art of  
20 experimental immunology and summarized in the  
Methods section, above. These include:  
monoclonal antibodies raised in mice against  
intact, purified protein from human fibroblasts;  
polyclonal antibodies raised in rabbits against  
25 synthetic peptides with sequences based on amino  
acid sequences predicted from the KGF cDNA  
sequence; polyclonal antibodies raised in  
rabbits against both naturally secreted KGF from



human fibroblasts and recombinant KGF produced in *E. coli* (see next section).

Monoclonal antibodies from three different hybridomas have been purified. All three recognize the recombinant as well as the naturally occurring KGF in a solid-phase (ELISA) assay. None cross-reacts with KGF under denaturing conditions (in a Western blot), and none neutralizes mitogenic activity of KGF in the BALB/MK bioassay.

Polyclonal antibodies were generated with a synthetic peptide with the amino acid sequence NDMTPEQMATNVR, corresponding to residues numbered 32 through 44 in KGF (see Fig. <sup>7</sup>~~II-1~~), plus an R (<sup>ARG</sup>~~arg~~) residue instead of the actual <sup>ASN</sup>~~asp~~ residue encoded by the cDNA at position 45. The <sup>ASN</sup>~~asp~~ residue is probably glycosylated in the natural KGF polypeptide and, therefore, <sup>appeared</sup>~~was not~~ identified to be an <sup>arg</sup>~~arg~~ in the amino acid sequencing data obtained directly from that polypeptide (see Discussion, below). Polyclonal antibodies generated with this synthetic peptide recognize both naturally occurring and recombinant KGF in ELISA and Western blot analyses at a level of sensitivity of at least as low as 10 ng protein. These antibodies, however, do not neutralize mitogenic activity of KGF in the BALB/MK bioassay.

Polyclonal antisera against intact natural KGF protein recognizes KGF in both ELISA and Western blot assays. Such antibodies also appear to inhibit mitogenic activity of KGF in the BALB/MK bioassay.

Expression of KGF cDNA in *E. coli*. KGF cDNA was expressed to produce polypeptide in *E. coli* by placing its coding sequence under control of the hybrid trk promoter (comprising elements of trp and lac promoters), in the plasmid pKK233-2 <sup>Amman, E. and Brosius, J., A&S, Here 40,183</sup> (II-31). To accomplish this, a specific length of KGF cDNA that contained the information to code for the mature KGF molecule (i.e., without its signal peptide) was amplified using the <sup>sub B14</sup> polymerase chain reaction technique (II-32). The fragment was directionally inserted between two sites in the vector, namely the *Nco*I site, made blunt ended by *S*1 nuclease digestion, and the *Hind*III site, using standard recombinant DNA methodology. Selected recombinants were sequenced at their cDNA 5' ends to ensure correct alignment of the ATG initiation codon with the regulatory elements of the trk promoter.

Several recombinants were tested for protein production by the usual small scale methods. In brief, the clones were grown to mid-exponential phase ( $OD_{595} \sim 0.5$ ), treated with 1 mM

isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) for 90 minutes, and cell extracts were run on SDS-polyacrylamide gels for Western blot analysis.

All recombinants tested synthesized a protein that was recognized by antibodies raised against an amino-terminal KGF peptide. One recombinant was selected which showed the greatest induction from IPTG, for further protein analyses.

One liter of bacteria was grown up in NZY broth containing 50  $\mu$ g/ml ampicillin and 12.5  $\mu$ g/ml tetracycline, to OD<sub>595</sub> 0.5, and treated for 90 min. with IPTG. The cells were collected by centrifugation, resuspended in 50 mM sodium phosphate (pH 7.3), 0.2 M NaCl, and lysed by sonication. Cell debris was removed by centrifugation, and lysate applied directly to a heparin-Sepharose affinity column.

As determined by Western blot analysis and mitogenic activity in keratinocytes, recombinant KGF was eluted in 0.5-0.6 M NaCl. Subsequent purification of the HSAC material with a Mono-S (FPLC) column (Pharmacia) yielded a preparation of KGF estimated to be  $\geq 90\%$  pure, as judged by electrophoretic analysis using SDS-polyacrylamide gels and silver-staining.

Recombinant KGF efficiently stimulated thymidine incorporation into BALB/MK keratinocyte cells, but was only marginally active on NIH/3T3

fibroblasts. Half-maximal stimulation of the BALB/MK cells in the standard keratinocyte bioassay was achieved with a concentration of between 2 to 5 ng/ml, compared to a concentration of 10 to 15 ng/ml for KGF purified from M426 cells. One liter of bacterial cells yielded approximately 50 µg of Mono-S purified recombinant KGF.

Construction of a chimera containing KGF

and aFGF sequences. The studies above indicated that KGF possessed two distinctive characteristics which might be encoded by distinct portions or domains of the polypeptide sequence, as is well known to occur in coding sequences of other multifunctional polypeptides. To test this possibility, a chimeric DNA segment encoding the NH<sub>2</sub>-terminal sequence of KGF grafted onto the C-terminal core of aFGF was constructed, as follows. A <sup>Sau3AI</sup> ~~Sau3AI~~ restriction enzyme site <sup>CCTGAGG</sup> (GATC) in the 5' end of the KGF cDNA, within codons for residues <sup>76, 77, and 78 (Tyr, Leu and Arg)</sup> ~~78 and 79 (arg and ile,~~ respectively; see Fig. II-1) was cut and joined to an homologous site in the aFGF cDNA within codons for amino acids <sup>37(Phe) 38(Leu) and 39(Arg)</sup> ~~39 (arg) and 40.~~ The 3' and 5' ends of this chimeric DNA were joined to the vector DNA of the plasmid pKK233-2 by the same method used for insertion of the KGF cDNA

encoding the secreted form of polypeptide (see Methods, above).

When recombinant *E. coli* cells were constructed using the vector carrying the chimera, and expression tests were conducted as described for mature KGF, above, a novel product with properties of both KGF and aFGF was produced. The peptide was enriched by heparin-Sepharose chromatography and found to have a target cell preference for keratinocytes, like KGF, with minimal activity on fibroblasts (NIH/3T3). The mitogenic activity of this chimeric polypeptide lacks, however, susceptibility to inhibition by heparin, a characteristic which parallels that of aFGF rather than KGF. In fact, the mitogenic activity on keratinocytes is actually enhanced by heparin, as is the case for aFGF. Thus the peptide domains responsible for <sup>target</sup> cell target specificity and heparin sensitivity are clearly distinct and readily separable in KGF, according to the practice of the present invention.

#### DISCUSSION

The experiments described in this section illustrate the practice of several principal embodiments of the present invention. These include isolation of cDNAs encoding KGF,

expression of such cDNAs in recombinant cells,  
production of various antibodies reactive with  
KGF, and construction and expression of a  
chimeric cDNA encoding a novel growth factor with  
5 amino acid sequences and related functionalities  
of both KGF and aFGF. The following points  
related to these embodiments may also be noted to  
enhance the understanding of the present  
invention.

10           The sequence predicted from the KGF cDNA  
agreed with the amino acid sequence determined  
from the purified KGF form secreted by human  
fibroblasts. Moreover, the sequence offered  
potential explanations for positions where  
15 definitive amino acid assignments could not be  
made by direct amino acid sequencing. Residues  
32 and 46 are predicted from the cDNA sequence to  
be cysteines, and hydrolyzed derivatives of  
unmodified cysteine residues are not detectable  
20 following Edman degradation. The predicted KGF  
amino acid sequence also contained one potential  
N-linked glycosylation site (Asn-X-Ser/Thr) from  
residues 45 through 47. If Asn 45 were  
glycosylated, it would not be detected by the  
25 amino acid sequencing methods employed here. In  
fact, KGF migrates as a broad band on  
NaDodSO<sub>4</sub>/PAGE at a higher molecular weight than

predicted for the purified protein. This may be accounted for by glycosylation.

The FGFs are heparin-binding mitogens

with broad target cell specificities (II-10).

~~FGF-5 and hst are transforming genes originally detected~~  
~~The hst gene was identified as a transforming gene~~  
~~by DNA mediated gene transfer.~~  
~~from a human stomach tumor (II-11), adjacent~~

~~normal stomach tissue (II-12), and from Kaposi's~~  
~~sarcoma (II-13), by standard NIH/3T3 transfection~~

assays. The product of the *int-2* gene is expressed normally during mouse embryogenesis (II-14) and

aberrantly after proviral integration of mouse mammary tumor virus (II-15).

KGF is the sixth member of the

fibroblast growth factor family to be identified

While the name FGF-6 does not seem

suitable because KGF is devoid of activity on

fibroblasts, this nomenclature may also be used

for this growth factor, to denote its structural

relationship to the FGF family. As all

previously characterized growth factors either

exclude epithelial cells as targets or include

them among a number of sensitive target cells,

the highly specific nature of KGF mitogenic

activity for epithelial cells, and the

sensitivity of keratinocytes in particular, make

it unique.

In studies to date, expression of the KGF transcript appears to be specific for stromal cells derived from epithelial tissues, suggesting its function in normal epithelial cell proliferation. The availability of the KGF cDNA clone will make it possible to determine whether abnormal expression of this growth factor can be implicated in clinical conditions characterized by epithelial cell dysplasia and/or neoplasia. Moreover, the ability to produce large quantities of this novel growth factor by recombinant techniques should allow testing of its clinical applicability in situations where specific growth of epithelial cells is of particular importance.

Alignment of the KGF sequence with the five other proteins of the FGF family revealed two major regions of homology, spanning amino acids 65-156 and 162-189 in the predicted KGF sequence, which were separated by a short, nonhomologous series of amino acids with varying lengths in different members of the family (Fig. <sup>1</sup>~~1~~<sub>2</sub>). In the case of *int-2*, the length of this sequence was 17 residues, while in *hst*, the two homologous regions were contiguous. In KGF the intervening sequence consisted of five amino acids.



In the aligned regions, the KGF amino acid sequence was about 44% identical to *int-2* (41% identical to FGF-5 (human) (mouse)), 39% identical to bFGF (human), 37% identical to aFGF (human) and 33% identical to *hst* (human). In this same region, all six proteins were identical at 19% of the residues, and allowing for conservative substitutions, they showed 28% homology.

As shown in Fig. II-2<sup>9</sup>, the amino termini of these related proteins are nonhomologous and of variable length. The primary KGF<sup>9</sup> and *hst* translation products contain hydrophobic

N-terminal regions which likely serve as signal sequences (II-16). The fact that this N-terminal domain is not present in the mature KGF molecule (Fig. II-1B<sup>7</sup>) further supports this conclusion.

In contrast, the FGFs are synthesized apparently without signal peptides (II-10). The *int-2* protein

contains an atypically short region of N-terminal hydrophobic residues (II-17), but it is not known

if the protein is secreted. Moreover, the *int-2* protein contains a long C-terminal extension compared to the other family members.

Purified KGF contains five cysteine residues, two of which are conserved throughout the family of FGF related proteins (Fig. II-2<sup>9</sup>). Also of note are the five pairs of basic residues

throughout the KGF sequence. This same pattern has been observed in other FGF family members and

may be involved in their interaction with heparin

*Schwamhauser, J.E., Tomlin, J.M., Lemischka, I.R. and Hynes, R.O., 1983, Cell 35, 421-431 (II-18).* Dibasic sites are also common targets

5 for proteolytic processing and such processing might account for the microheterogeneity observed in some KGF preparations (unpublished data).

The KGF cDNA sequence was AT rich throughout its length, but particularly so in the  
10 3' untranslated region where the AT content was 70% as compared to 60% in the putative coding sequence and 63% in the 5' untranslated region. The 3' untranslated region contained a large number of ATTTA sequences, which have been  
15 proposed to be involved in the selected

degradation of transiently expressed, unstable  
8 *Staw, M. and Kover, R., 1986, Cell 46, 659-667 (II-19).* There was no classical AATAAA

polyadenylation signal but two variant sequences,  
8 *Birnsteil, M.L., Busslinger, M. and Strub, K., 1985, Cell 41, 349-359 (II-20),* were detected 24 and

20 19 nucleotides, respectively, upstream of the poly(A) sequence at the 3' end of the cDNA.

It has been suggested that the heparin effect on acidic FGF is either due to stabilization of the active conformation of the

25 growth factor or to formation of a tertiary

*Schiefer, A.B., Kenny, J., Kowalski, W., Friesel, J., Melton, T. and Maciag, T., 1985, Proc. Natl. Acad. Sci. USA 82, 6138-6142 (II-21),*  
*Rogozdarowicz, O. and Cheng, J., 1986, J. Cell Physiol. 128, 475-485 (II-22).* If so, heparin may stabilize a

conformation of KGF that is not as active as the

free molecule, or form a tight complex that is unable to efficiently interact with its receptor.

While its ability to bind heparin reflects the structural similarities of KGF with the FGF's, the differences in target cell specificities between these related mitogens is remarkable. The FGF's induce division of most nonterminally differentiated cells of both embryonic mesodermal and neuroectodermal origin.

10 In addition to fibroblasts and vascular endothelial tissues, mesodermally derived targets

in culture include myoblasts, chondrocytes and osteoblasts (II-23). FGF's are also mitogenic for glial astrocytes and neuroblasts (II-24).  
*Theras, K.A. and Himelney-Mallago, H., 1986, Trends Biochem. Sci. 11, 81-84*  
*Hensburger, C., Sabotindelle, H. and Hensebrenner, M., 1987, FEBS Lett. 217, 1-5*

15 The product of the oncogene isolated from Kaposi's sarcoma, which is identical to *hst*, also

stimulates proliferation of NIH/3T3 and capillary endothelial cells (II-25). To date, KGF induced  
*Delli-Bovi, P., Cristofa, A.M., Kain, F.G., Mico, A., Litton, M. and Basilico, C., 1987, Cell 50, 729-737*

mitogenesis has only been observed in epithelial cells, and the absence of any detectable activity in fibroblasts or endothelial cells has also been demonstrated (see Experimental Section I, above and II-3). It seems likely, therefore, that KGF acts through a different cell surface receptor than the FGFs.

There is no significant N-terminal homology between KGF and other FGF-related proteins. Thus, the construction of chimeric

molecules between KGF and a prototype FGF was undertaken to determine whether the KGF N-terminal domain is sufficient to account for its unique target cell specificity. The results on the first such recombinant polypeptide sequence indicate that the N-terminal domain of KGF essentially encodes the cell preference for keratinocytes, while the susceptibility of KGF to heparin is encoded somewhere in the C-terminal core region which was replaced by sequences of aFGF. This novel KGF-like growth factor may have advantages in clinical applications where administration of an epithelial-specific growth factor is desirable in the presence of heparin, a commonly used anticoagulant. Additional studies on chimeras should also provide insights into which specific domains in the C-terminal core contribute the different effects of heparin on their biologic activities.

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For purposes of completing the  
background description and present disclosure,  
each of the published articles, patents and  
patent applications heretofore identified in this  
specification <sup>is</sup> ~~are~~ hereby incorporated by  
reference into the specification.

The foregoing invention has been  
described in some detail for purposes of clarity  
and understanding. It will also be obvious that  
various combinations in form and detail can be  
made without departing from the scope of the  
invention.